ADNI Alzheimer's Disease Neuroimaging Initiative

Method Validation Report for a Selected Subset of 24 Phosphatidylcholine (PtdCho) and 10 Lysophosphatidylcholine (LPtdCho) Species using Negative Ionization Stable Isotope Dilution, Flow Injection Tandem Mass Spectrometry

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On behalf of: The Alzheimer's Disease Metabolomics Consortium (ADMC)

Disclaimer: This method validation report contains basic validation protocols and results necessary for suitability determination for investigational research use only. For greater clarity, this method and results generated by this method are to be used for non-regulated purposes only.

phenomenome discoveries

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Method Summary

The following report describes the materials and procedures used to quantitate 24 phosphatidylcholine (PtdCho) and 10 lysophosphatidylcholine (LPtdCho) glycerophospholipids species. The method has triplicate redundancy in that 8 sn-2 fatty acid compositions (18:0, 18:1, 18:2, 18:3, 20:4, 20:5, 22:4, 22:6) are measured on the 3 most conserved sn-1 compositions (16:0, 18:0, 18:1) except for 18:0/18:1 which cannot be discriminated from 18:1/18:0. 16:1/22:6 is included as an extra measurement of DHA containing PtdCho. These 10 fatty acid moieties are also measured as LPtdCho. All 24 endogenous PtdCho species are quantitated using a single stable isotope dilution curve. This standard curve is generated using a constant concentration of non-endogenous $^{2}H_{31}$-PtdCho 16:0/18:1 species and varying concentrations of the endogenous PtdCho 16:0/18:1 species. An equivalent constant concentration of $^{2}H_{31}$-PtdCho 16:0/18:1 is added to all samples prior to sample preparation. Serum concentrations are determined by extrapolating the observed isotope ratio from the theoretical standard curve. Accordingly, all results are expressed as PtdCho 16:0/18:1 equivalents. Although precision is empirically measured for all species, accuracy can only be measured for PtdCho 16:0/18:1 and assumed to be equivalent for the other 23 species. The same procedure is used for quantitating the 10 LPtdCho species using $^{2}H_{35}$-LPtdCho 18:0 and LPtdCho 18:0 as the non-endogenous and endogenous isotopes, respectively.

Proteins and non-polar analytes are extracted from serum using a pH adjusted liquid/liquid extraction procedure. All the choline analytes are converted into their respective formate adducts using 75 mM AmF as a buffer in the extraction solvent to improve the NESI detection. Analyte intensities are measured by direct injection of the remaining aqueous solution into an API-4000 tandem mass spectrometer operating in the negative ionization electrospray (ESI) mode. For all PtdCho and LPtdCho analytes, the (M+COO-H)- ion is selected by Q1 to be fragmented in the collision cell and the sn-1/2 (R-COO-) ion is selected by Q3 and used as the daughter ion.

Method performance characteristics of recovery (89-101% and 89-120% for PtdCho and LPtdCho, respectively), accuracy (82-118% and 88-114% for PtdCho and LPtdCho, respectively), and within run precision (2-8% and 2-5% for PtdCho and LPtdCho, respectively) are such that the method meets basic validation criteria for investigational, case-control, regulatory-exempt, research use.

Materials required

- *Pipettes* – Eppendorf Reference Pipettes, 20µL, 100µL, 200µL, 1000µL (Eppendorf; Hamburg, Germany)
- *5, 15, and 150 ml Falcon Tubes* or equivalent
- *Centrifuge* – Sufficient size to centrifuge two 96 well plates at 2000RCF
- *Bottle top Dispenser* – ChemSaver™ bottle top dispenser or equivalent (1-10 mL or 0.5-2.5 mL) (Brinkmann Instruments Inc; Westbury, NY, USA)

- *20µL Barrier Pipet Tips* – VWR - Cat#: 87001-694 or equivalent
- *Matrix 1.4mL Polypropylene Round Bottom Tubes*- (Fisher Scientific) Cat#50823880 or equivalent
- *Matrix 1.4mL Rack* – (Thermo Fisher); Cat# 50823921 or equivalent
- *Matrix Cap Strips (EVA)* – (Thermo Fisher); Cat# 50823825 or equivalent
- *Vortex Mixer* – Vortex-Genie I™ (Scientific Industries Inc; Bohemia, NY, USA) or equivalent
- Mechanical stirrer (VWR DVx2500 Multi-Tube Vortex) or equivalent
- *Thermo Scientific Unmarked Analysis Tubes* (Fisher Scientific) Cat# AB4170 or equivalent
- *Thermo Scientific Matrix SepraSeal Capping System; Pre – Split; Piercable; Autoclavable Caps* (Fisher Scientific) Thermo No. 4465BLU
- *HPCL Glass Vial* – Chromatographic Specialties, Cat# CS51820714
- *HPCL Vial Cap* – Chromatographic Specialties, Cat# CS51820717

Solvents and reagents:

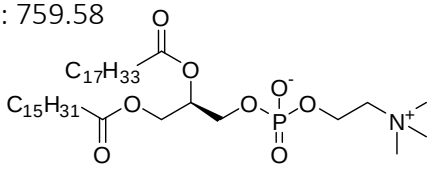
- *Methanol* – Optima Grade, cat. A4544, (Fisher Scientific) or equivalent
- *Ammonium Formate* – cat. 17843-250G, (Sigma Aldrich Canada Ltd.) or equivalent
- *Ultrapure Water* – HPLC Grade Water, cat. W7-4 (Fisher Scientific) or equivalent
- *Hexanes* – H292-4 (Fisher Scientific) or equivalent

Standards:

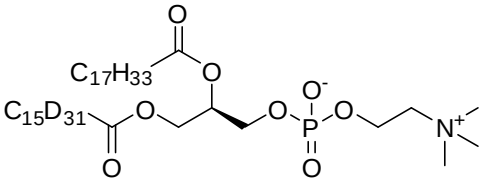
- 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PtdCho (16:0/18:1) purchased from Avanti Polar Lipids Inc. (Alabaster, AL)
- 1-palmitoyl-(31D) -2-oleoyl-sn-glycero-3-phosphocholine (31D-PtdCho (16:0/18:1) purchased from Avanti Polar Lipids Inc. (Alabaster, AL)
- 1-stearoyl-sn-glycero-3-lysophosphocholine (Lyso-PtdCho (18:0/OH) purchased from Avanti Polar Lipids Inc. (Alabaster, AL)
- 1-stearoyl (35D)-sn-glycero-3-lysophosphocholine (35D-Lyso-PtdCho (18:0/OH) purchased from Avanti Polar Lipids Inc. (Alabaster, AL)

Standard structures:

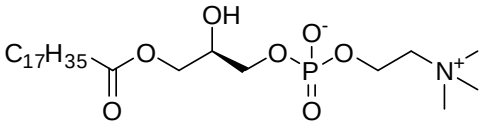
PC Standard: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Chemical Formula: $C_{42}H_{82}NO_8P$, Exact Mass: 759.58



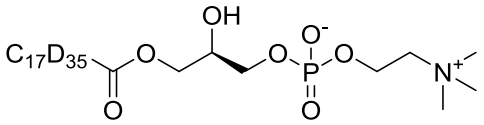
Deuterated PC Standard: 1-palmitoyl-(31D) -2-oleoyl-sn-glycero-3-phosphocholine: Chemical Formula: $C_{42}H_{51}D_{31}NO_8P$, Exact Mass: 790.77



LPC Standard: 1-stearoyl-sn-glycero-3-lysophosphocholine, Chemical Formula: $C_{26}H_{54}NO_7P$, Exact Mass: 523.36



Deuterated LPC Standard: 1-stearoyl (35D)-sn-glycero-3-lysophosphocholine: Chemical Formula: $C_{26}H_{19}D_{35}NO_7P$, Exact Mass: 558.58



LC-MS/MS conditions

Mass Spectrometer – API 4000 triple quadrupole mass spectrometer equipped with a negative ESI source.
Agilent 1100 HPLC pump, Agilent 1200 series auto sampler equipped with automated sample injection and solvent delivery pump.

- LC flow rate: 0.5ml/min
- Mobile phase: 5% water in methanol (MeOH)
- Acquisition time: 2.0min/sample
- MS Source Parameter:

Description	Value
Scan Type	MRM
Polarity	Negative
Curtain Gas	35 V
Source Temperature	400 ºC
Source Gas 1 (GS 1)	50 V
Source Gas 2 (GS2)	55 V
Collision Gas (CAD)	8 V
Ion Spray Voltage (IS)	4500 V
Dwell time	50 msec

36MRMs Analysis transition:

MRM #	Metabolite Name	Molecular Formula	Parent Mass	(M+FA-H) Mass	Fragment Mass	MRM Transition
1	GPCho 16:0/18:3	C42H78NO8P	755.5465	800.6	(C18H29O2) - 277.2	800.6 / 277.2
2	GPCho 16:0/18:0	C42H84NO8P	761.5935	806.6	(C16H31O2) - 255.2	806.6 / 255.2
3	GPCho 16:0/18:1	C42H82NO8P	759.5778	804.6	(C18H33O2) - 281.2	804.6 / 281.2
4	GPCho 16:0/18:2	C42H80NO8P	757.5622	802.6	(C18H31O2) - 279.2	802.6 / 279.2
5	GPCho 16:0/20:4	C44H80NO8P	781.5622	826.6	(C20H31O2) - 303.2	826.6 / 303.2
6	GPCho 16:0/20:5	C44H78NO8P	779.5465	824.6	(C20H29O2) - 301.2	824.6 / 301.2
7	GPCho 16:0/22:4	C46H84NO8P	809.5935	854.6	(C22H35O2) - 331.3	854.6 / 331.3
8	GPCho 16:0/22:6	C46H80NO8P	805.5622	850.6	(C22H31O2) - 327.3	850.6 / 327.3
9	GPCho 16:1/22:6	C46H78NO8P	803.5465	848.6	(C22H31O2) - 327.3	848.6/327.3
10	GPCho 18:0/18:0	C44H88NO8P	789.6248	834.6	(C18H35O2) - 283.2	834.6 / 283.2
11	GPCho 18:0/18:1	C44H86NO8P	787.6091	832.6	(C18H33O2) - 281.2	832.6 / 281.2
12	GPCho 18:0/18:2	C44H84NO8P	785.5935	830.6	(C18H31O2) - 279.2	830.6 / 279.2
13	GPCho 18:0/18:3	C44H82NO8P	783.5778	828.6	(C18H29O2) - 277.2	828.6 / 277.2
14	GPCho 18:0/20:4	C46H84NO8P	809.5935	854.6	(C20H31O2) - 303.2	854.6 / 303.2
15	GPCho 18:0/20:5	C46H82NO8P	807.5778	852.6	(C20H29O2) - 301.2	852.6 / 301.2
16	GPCho 18:0/22:4	C48H88NO8P	837.6248	882.6	(C22H35O2) - 331.3	882.6 / 331.3
17	GPCho 18:0/22:6	C48H84NO8P	833.5935	878.6	(C22H31O2) - 327.3	878.6 / 327.3
18	GPCho 18:1/18:1	C44H84NO8P	785.5935	830.6	(C18H33O2) - 281.2	830.6 / 281.2
19	GPCho 18:1/18:2	C44H82NO8P	783.5778	828.6	(C18H31O2) - 279.2	828.6 / 279.2
20	GPCho 18:1/18:3	C44H80NO8P	781.5622	826.6	(C18H29O2) - 277.2	826.6 / 277.2
21	GPCho 18:1/20:4	C46H82NO8P	807.5778	852.6	(C20H31O2) - 303.2	852.6 / 303.2
22	GPCho 18:1/20:5	C46H80NO8P	805.5622	850.6	(C20H29O2) - 301.2	850.6 / 301.2
23	GPCho 18:1/22:4	C48H86NO8P	835.6091	880.6	(C22H35O2) - 331.3	880.6 / 331.3
24	GPCho 18:1/22:6	C48H82NO8P	831.5778	876.6	(C22H31O2) - 327.3	876.6 / 327.3
25	31d_GPCho 16:0/18:1	C42H51NO8PD31	790.7724	835.8	(C16D31O2) - 286.3	835.8 / 286.3
26	LGPCho 16:0	C24H50NO7P	495.3	540.3	(C16H31O2) - 255.2	540.3 / 255.2
27	LGPCho 16:1	C24H48NO7P	493.3	538.4	(C16H29O2) - 253.2	538.3 / 253.2
28	LGPCho 18:0	C26H54NO7P	523.4	568.4	(C18H35O2) - 283.2	568.4 / 283.3
29	LGPCho 18:1	C26H52NO7P	521.3	566.3	(C18H33O2) - 281.2	566.3 / 281.2
30	LGPCho 18:2	C26H50NO7P	519.3	564.3	(C18H31O2) - 279.2	564.3 / 279.3
31	LGPCho 18:3	C26H48NO7P	517.3	562.3	(C18H29O2) - 277.2	562.3 / 277.3
32	LGPCho 20:4	C28H50NO7P	543.3	588.3	(C20H31O2) - 303.2	588.3 / 303.2
33	LGPCho 20:5	C28H48NO7P	541.3	586.3	(C20H29O2) - 301.2	586.3 / 301.2
34	LGPCho 22:6	C30H50NO7P	567.3	612.3	(C22H31O2) - 327.3	612.3 / 327.2
35	LGPCho 22:4	C30H54NO7P	571.4	616.4	(C22H35O2) - 331.3	616.3 / 331.2
36	35d_LGPCho (18:0/OH)	C26H19NO8PD35	558.5835	603.6	(C18D35O2) - 318.5	603.6 / 318.5

Sample preparation

These quantities are based on 96 sample extractions using Thermo 1.4 ml matrix tubes.

Preparation of stable isotope internal standard solution

1. Prepare 31D-PtdCho (16:0/18:1) and 35D-LPtdCho (18:0/OH) deuterated standard stock solution (IS Stock B). Use 100 µg/ml concentration of each deuterated PtdCho standard by adding 100 µl of each 1 mg/ml deuterated standard to 800 µl of 75 mM AmF in 10% water, 90% MeOH prepared in step a (see table below).

Stock Name	Standard	1 mg/ml	MeOH (ul)	Conc (ug/ml)
IS Stock B	31D-PtdCho(16:0/18:1)	100	800	100
	35D-L-PtdCho(18:0/OH)	100		100

2. Prepare 60 ml of 75 mM ammonium formate (AmF) solution using a 10% water, 90% methanol solution in a 150ml falcon tube according to the table below:

AmF (mg)	Water (ml)	MeOH (ml)	AmF (mM)
283.77	6	54	75

   a. Weigh the given amount of AmF into a 150 ml falcon tube.

   b. Add 6.0 ml of HPLC grade water from a dispenser pump into the tube, and vortex until all of the AmF solids are dissolved.

   c. Add 54.0 ml (6 x 9 ml) of methanol into each tube using a solvent dispenser pump. Vortex until all the components are mixed well.

3. Remove 375.0 µl from the above prepared solution and discard. This is to make up the difference for standards being added in the next step.

4. Add 375.0 µl of IS Stock B (prepared above in Step 1) solution into the falcon tube.

5. Vortex the 150 ml falcon tube. The tube (IS Stock C) should now contain both standards at a final concentration of 0.625 µg/ml and will be used as the standard curve diluent (3.2 step 4 and 5) and in the sample extraction (3.3 step 2).

Preparation of the external isotope dilution curve (IDC)

The standard curve consists of nine concentrations plus a zero point as shown in the table below.

CC #	PtdCho(16:0/18:1) ug/ml	L-PtdCho(18:0/OH) ug/ml	31D-PhtCho(16:0/18:1) ug/ml	35D-L-PhtCho(18:0/1OH) ug/ml
CC_09	5.00	5.00	0.625	0.625
CC_08	2.50	2.50	0.625	0.625
CC_07	1.25	1.25	0.625	0.625
CC_06	0.63	0.63	0.625	0.625
CC_05	0.31	0.31	0.625	0.625
CC_04	0.16	0.16	0.625	0.625
CC_03	0.08	0.08	0.625	0.625
CC_02	0.04	0.04	0.625	0.625
CC_01	0.02	0.02	0.625	0.625
CC_00	0.00	0.00	0.625	0.625

1. Prepare PtdCho (16:0/18:1) and L-PtdCho (18:0/OH) 12C standard curve stock solution (PC/LPC Stock A) by adding 50 µl of each 12C standard (at 1 mg/ml) to 400 µl of 75 mM AmF in 10% water, 90% MeOH (see table below).

Stock Name	Standard	img/ml veolume/ul	MeOH (ul)	Conc (ug/ml)
PC/LPC Stock A	PtdCho(16:0/18:1)	50	400	100
	L-PtdCho(18:0/OH)	50		100

2. Prepare 10 ml of 75 mM ammonium formate (AmF) using a 10% water, 90% methanol solution in a 15ml falcon tube according to the table below:

AmF (mg)	Water (ml)	MeOH (ml)	AmF (mM)
283.77	6	54	75

   a. Weigh the given amount of AmF into a 15 ml falcon tube.

   b. Add 2.0 ml of HPLC grade water from a dispenser pump into the tube, and vortex until all of the AmF solids are dissolved.

   c. Add 18.0 ml of methanol using a solvent dispenser pump into the above tube. Vortex until all the components are mixed well.

3. Prepare 2.0 ml of the highest concentration curve-point (CC-09) by combining the following in an 1.5 ml Eppendorf tube:

   - 100 µl of PC/LPC Stock A (3.2 step 1).
   - 12.5 µl of IS Stock B (3.1 step 1).
   - 1875 µl AmF in 10% water, 90% MeOH (3.3 step 2).

CC #	PC/LPC Stock A Volume (ul)	IS Stock B Volume (ul)	75 mM AmF Solution (ul)	Conc of PtdCho(16:0/18:1) (ug/ml)	Conc of L-PtdCho(18:0/OH) (ug/ml)	Conc of 31D-PtdCho(16:0/18:1) (ug/ml)	Conc of 35D-L-PtdCho(18:0/OH) (ug/ml)
CC_09	100	12.5	1887.5	5	5	0.625	0.625

4. Create the remaining standard curve dilutions by diluting 500 µl of CC_09 with 500 µl of the 0.625 µg/ml IS stock C solution (3.1 step 2-5) to create 1000 µl CC-08.

5. Repeat the above step by diluting CC_08 to create CC_07. Repeat until all curve points to CC_01 are complete according to the table below.

CC #	CC_# volume/ul	IS Stock C Volume / ul	PtdCho(16:0/18:1) ug/ml	L-PtdCho(18:0/OH) ug/ml	31D-PhtCho(16:0/18:1) ug/ml	35D-L-PhtCho(18:0/1OH) ug/ml
CC_09	1000	0.00	5.00	5.00	0.625	0.625
CC_08	500.0 of CC_09	500.0	2.50	2.50	0.625	0.625
CC_07	500.0 of CC_08	500.0	1.25	1.25	0.625	0.625
CC_06	500.0 of CC_07	500.0	0.63	0.63	0.625	0.625
CC_05	500.0 of CC_06	500.0	0.31	0.31	0.625	0.625
CC_04	500.0 of CC_05	500.0	0.16	0.16	0.625	0.625
CC_03	500.0 of CC_04	500.0	0.08	0.08	0.625	0.625
CC_02	500.0 of CC_03	500.0	0.04	0.04	0.625	0.625
CC_01	500.0 of CC_02	500.0	0.02	0.02	0.625	0.625
CC_00	0	500.0	0.00	0.00	0.625	0.625

6. For the CC_00 point, use only 500 µl of the 0.625 µg/ml *IS stock C* solution.

Sample extraction preparation

1. Pipette 20 µl of each serum sample (93 maximum) into individual 1.4 ml ThermoFisher matrix tubes and arrange in a 96-position extraction rack with EQA (Extraction Quality Assurance) samples spaced at the beginning, middle, and end of the rack as follows:

IRQA 1	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35
S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47
S48	S49	S50	S51	S52	IRQA 2	S53	S54	S55	S56	S57	S58
S59	S60	S61	S62	S63	S64	S65	S66	S67	S68	S69	S70
S71	S72	S73	S74	S75	S76	S77	S78	S79	S80	S81	S82
S83	S84	S85	S86	S87	S88	S89	S90	S91	S93	S93	IRQA 3

2. Add 500 µl of the 0.625 µg/ml D-PC/D-LPC IS stock C solution (3.1step 2-5) into to each serum sample.
3. Add 400 µl of hexane to each serum sample.
4. Vortex the rack for 5 minutes at 1400 rpm.
5. Centrifuge the rack for 10 min at 3500 rpm in a Beckman bench top centrifuge.

Analysis run set up

This method uses an Applied Biosystems API4000 triple quadrupole mass spectrometer in negative ESI coupled to an Agilent 1500 HPLC system to introduce the sample by direct flow injection. The sample configuration consists of two 54-vial racks, with samples bracketed by the standard curve samples as shown below:

Rack 1

	1	2	3	4	5	6	7	8	9
A	Bl 1	S3	S9	S14	S20	S25	S31	CC 04	S42
B	CC 00	S4	S10	S15	S21	S26	S32	S37	S43
C	CC 01	S5	S11	S16	S22	S27	S33	S38	S44
D	EQA01	S6	S12	S17	S23	S28	S34	S39	S45
E	S1	S7	CC 02	S18	S24	S29	S35	S40	S46
F	S2	S8	S13	S19	CC 03	S30	S36	S41	EQA02

Rack 2

	1	2	3	4	5	6	7	8	9
A	S 47	S52	S58	S63	S69	S74	S80	S85	S91
B	CC 05	S53	S59	S64	S70	S75	S81	S86	S92
C	S 48	S54	CC 06	S65	S71	S76	S82	S87	S93
D	S 49	S55	S60	S66	CC 07	S77	S83	S88	EQA03
E	S 50	S56	S61	S67	S72	S78	CC 08	S89	CC 09
F	S 51	S57	S62	S68	S73	S79	S84	S90	Bl 2

Bl = Blank CC = Calibration Curve Blank EQA = QA Sample S = Sample

1. Transfer 125 µl of the bottom (aqueous) layer (prepared in 3.3) into a plastic insert placed in an HPLC vial.

2. Arrange the method blanks (solution prepared in 3.3 step 2), calibration curve points (prepared in 3.2) and samples according to the given sample rack layout.

Data analysis

The 3Q-NESI serum phosphocholine quantitation method is a flow injection method that utilizes chromatography. The peak area for each analyte under the given MRM transition is individually integrated using a quantitative method built up using "Quantitation Wizard" in the "Analyst" software in the AB Sciex API 4000 Mass Spectrometer. The concentration in nano mole (nm)/ml for each analyte is obtained using the following steps.

1. The IS ratios for each analyte is obtained using the peak area ratio of each endogenous analyte to the spiked in deuterated standard; such as each endogenous PtdCho/31D PtdCho (16:0/18:1) and endogenous Lyso-PtdCho/35DL-PtdCho(18:0/OH).

2. The IS ratios for each analyte is converted into µg/ml concentrations using the external IDC linear equation and consequently converted into nm/ml concentrations using n = m/Mm equation.

Quality assurance

Quality control samples (extraction quality assurance-EQA sampled)

Three QC samples (EQA_01, EQA_02, and EQA_03) of pooled human serum are run at the beginning, middle, and end in each batch of 93 patient samples to track the run acceptability.

The run is accepted if each of the replicate is less than 15% RSD within the run. After all the patient samples (833 in total) are run, each of the batch specific QA samples are statistically analyzed to check any batch variability (refer to QA/QC report).

Curve acceptance

The following criterion constitutes an acceptable calibration curve:

- % deviance of actual vs. nominal values for the two analytical standards, PtdCho (16:0/18:1) and L-PtdCho (18:0/OH) should be of less than 20% for CC_01 and less than 15% for CC_02 – CC_09.

If the calibration curve is not immediately acceptable, removal of no more than two standards from the standard curve and re-processing of the curve is allowed, as long as these points are not the LLoQ or the ULoQ. The removal of any curve point must be substantiated by means of an acceptable mathematical approach for removing outliers.

Method validation

Precision

Precision is determined using five independently prepared isotope dilution curves that run in a randomized order. Precision of the isotope ratio of two phosphocholine analytical standards is determined for individual concentrations by calculating the RSD (%CV) of their responses in the expected range of concentrations in 5 replicates. The mean should not exceed 15% CV, except for the LLOQ where 20% is the upper limit.

CC #	Conc (ug/ml)	L-PtdCho (18:0/OH)/35D-L-PtdCho(18:0/OH)_Precision					Ave	SD	%CV	L-PtdCho (18:0/OH)/35D-L-PtdCho(18:0/OH)_Precision					Ave	SD	%CV
		R1	R2	R3	R4	R5				R1	R2	R3	R4	R5			
CC_00	0.00	0.04	0.03	0.03	0.03	0.04	0.04	0.00	11%	0.01	0.02	0.02	0.02	0.01	0.01	0.00	16%
CC_01	0.02	0.67	0.77	0.67	0.63	0.67	0.68	0.05	8%	0.23	0.23	0.23	0.21	0.22	0.22	0.01	3%
CC_02	0.04	1.35	1.40	1.36	1.32	1.25	1.34	0.05	4%	0.42	0.45	0.44	0.43	0.41	0.43	0.01	3%
CC_03	0.08	2.59	2.68	2.53	2.56	2.53	2.58	0.06	2%	0.85	0.87	0.85	0.81	0.87	0.85	0.02	3%
CC_04	0.16	5.02	5.06	4.98	4.87	4.77	4.94	0.12	2%	1.64	1.61	1.55	1.61	1.60	1.60	0.03	2%
CC_05	0.31	9.89	10.15	10.05	9.76	9.20	9.81	0.37	4%	3.27	3.42	3.37	3.12	3.25	3.29	0.12	4%
CC_06	0.63	18.48	19.95	19.84	19.66	19.16	19.42	0.61	3%	6.20	6.47	6.23	6.25	6.27	6.28	0.11	2%
CC_07	1.25	35.64	38.33	37.46	36.29	37.09	36.96	1.04	3%	12.15	12.60	12.48	12.60	12.21	12.41	0.21	2%
CC_08	2.50	65.20	72.10	67.59	65.33	68.83	67.81	2.85	4%	21.45	24.14	24.22	23.33	22.48	23.13	1.17	5%
CC_09	5.00	116.18	127.79	127.99	114.42	117.86	120.85	6.54	5%	41.49	47.06	43.35	42.57	43.52	43.60	2.09	5%

Accuracy

Accuracy measures how close the test result is to the nominal value (concentration) of the analyte; it is described as a percentage of the test results compared with the nominal value. Accuracy is determined using five independently prepared isotope dilution curves that run in a

randomized order. The calculated mean at each concentration is compared to nominal values to determine the percent deviation of the mean from the nominal values, which serves as a measurement of the accuracy of the analytical method. The mean should not exceed 15% CV, except for the LLOQ where 20% is the upper limit.

CC #	Conc (ug/ml)	L-PtdCho (18:0/OH)/35D-L-PtdCho(18:0/OH)_Accuracy					Ave	SD	%CV	L-PtdCho (18:0/OH)/35D-L-PtdCho(18:0/OH)_Accuracy					Ave	SD	%CV
		R1	R2	R3	R4	R5				R1	R2	R3	R4	R5			
CC_00	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CC_01	0.02	119%	127%	112%	119%	115%	118%	0.058	5%	118%	119%	118%	108%	115%	116%	0.044	4%
CC_02	0.04	119%	116%	114%	125%	108%	116%	0.064	5%	110%	117%	111%	109%	106%	111%	0.040	4%
CC_03	0.08	114%	111%	107%	121%	109%	112%	0.059	5%	112%	114%	108%	103%	113%	110%	0.043	4%
CC_04	0.16	111%	104%	105%	115%	102%	108%	0.055	5%	107%	105%	99%	102%	104%	103%	0.030	3%
CC_05	0.31	109%	105%	106%	116%	99%	107%	0.063	6%	107%	112%	107%	99%	105%	106%	0.046	4%
CC_06	0.63	102%	103%	104%	117%	103%	106%	0.061	6%	101%	106%	99%	99%	102%	101%	0.026	3%
CC_07	1.25	99%	99%	98%	107%	99%	101%	0.039	4%	99%	103%	100%	100%	99%	100%	0.016	2%
CC_08	2.50	90%	93%	89%	97%	92%	92%	0.030	3%	87%	99%	97%	93%	91%	93%	0.044	5%
CC_09	5.00	80%	82%	84%	85%	79%	82%	0.025	3%	85%	96%	87%	85%	88%	88%	0.048	5%

Recovery

Recovery measures the extraction efficiency for a given method within the defined limits of variability. It is determined by comparing the back-calculated extracted concentration to the nominal concentration and expressed as a percentage. Although there is no absolute acceptance criterion for recovery, typically, 70-130% recovery is required to be achieved for LC-MS/MS method. Low (0.025 µg/ml), medium (0.1 ug/ml), and high (1.0 µg/ml) concentrations of L-PtdCho (18:0/OH) and PtdCho (16:0/18:1) standards with constant amount of their deuterated standards (0.625 ug/ml) is extracted in solvent in 5 replicates. The peak area ratios of 12C std:IS is extrapolated from an external IDC prepared in the same solvent to obtain the percent recoveries of the 12C PC standards at each concentration.

PtdCho (16:0/18:1)	PtdCho (16:0/18:1)/35D-PtdCho (16:0/18:1)						% Rec_PtdCho (16:0/18:1)						
Conc(ug/ml)	R1	R2	R3	R4	R5	% CV	R1	R2	R3	R4	R5	Ave % Rec	% CV
0.025	0.28	0.30	0.30	0.30	0.32	5%	102%	112%	110%	112%	120%	111%	5%
0.10	0.95	0.84	0.91	0.96	0.89	5%	95%	83%	91%	96%	90%	91%	6%
1.00	10.24	8.45	8.89	9.52	8.83	8%	106%	87%	92%	98%	91%	95%	8%

L-PtdCho (18:0/OH)	L-PtdCho (18:0/OH)/351-L-PtdCho (18:0/OH)						% Rec_L-PtdCho (18:0/OH)						
Conc(ug/ml)	R1	R2	R3	R4	R5	% CV	R1	R2	R3	R4	R5	Ave % Rec	% CV
0.025	0.94	0.96	1.06	1.11	1.20	10%	93%	96%	109%	115%	128%	109%	13%
0.10	3.13	2.83	3.17	3.45	3.00	7%	95%	85%	96%	106%	91%	95%	8%
1.00	34.20	29.24	31.38	32.99	29.22	7%	112%	95%	102%	108%	95%	102%	7%

Low (0.02, 05 µg/ml), medium (0.625 µg/ml), and high (5.0 µg/ml) concentrations of one deuterated standard is spiked into serum while the other deuterated standard is spiked at a

constant concentration (0.625 µg/ml) and is extracted with serum in 5 replicates. The peak area ratios of D_choline:IS is extrapolated from the corresponding external IDC prepared in solvent to obtain the percent recovery (after background subtraction, if any) of the deuterated choline standard at each concentration.

31D_PtdCho (16:0/18:1)	31D_PC (16:0/18:1)_Observed Concentration ug/ml							31D_PtdCho (16:0/18:1)_% Rec						
Conc(ug/ml)	R1	R2	R3	R4	R5	Ave	% CV	R1	R2	R3	R4	R5	Ave	% CV
0.00	0.01	0.01	0.01	0.01	0.02	0.01	39%	N/A	N/A	N/A	N/A	N/A	N/A	N/A
0.05	0.07	0.05	0.04	0.06	0.04	0.05	21%	131%	98%	83%	112%	81%	101%	21%
0.63	0.59	0.57	0.53	0.47	0.61	0.56	10%	94%	92%	85%	75%	98%	89%	10%
5.00	4.37	5.41	4.03	4.88	4.40	4.62	12%	87%	108%	81%	98%	88%	92%	12%

35D_LPtdCho (18:0/OH)	35D_LPC (18:0/OH)_Observed Concentration ug/ml							35D_LPtdCho (18:0/OH)_% Rec						
Conc(ug/ml)	R1	R2	R3	R4	R5	Ave	% CV	R1	R2	R3	R4	R5	Ave	% CV
0.00	0.01	0.00	0.00	0.00	0.00	0.00	127%	N/A	N/A	N/A	N/A	N/A	N/A	N/A
0.05	0.04	0.05	0.06	0.06	0.07	0.06	20%	96%	94%	114%	123%	142%	114%	17%
0.63	0.62	0.71	0.70	0.91	0.83	0.75	15%	99%	114%	111%	145%	132%	120%	15%
5.00	4.97	4.61	4.17	4.42	4.14	4.46	8%	99%	92%	83%	88%	83%	89%	8%

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